



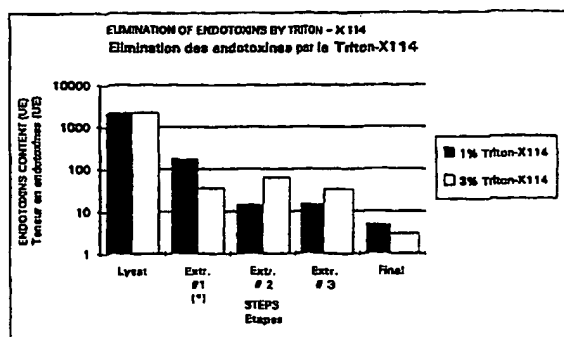
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(71) Déposant (pour tous les Etats désignés sauf US): TRANSGENE S.A. [FR/FR]; 11, rue de Molsheim, F-67000 Strasbourg (FR).			
(72) Inventeur; et (75) Inventeur/Déposant (US seulement): CAVALLINI, Bruno [FR/FR]; 13, rue Saint-Cécile, F-67100 Strasbourg (FR).			
(74) Mandataires: MARTIN, Jean-Jacques etc.; Cabinet Regimbeau, 26, avenue Kléber, F-75116 Paris (FR).			

(54) Title: METHOD FOR PREPARING A PLASMID DNA

(54) Titre: PROCEDE DE PREPARATION D'ADN PLASMIDIQUE



LEGEND  
 Légende : Lysat Lysat alcalin initial INITIAL ALKALINE LYSATE  
 Extr. 1(\*) Après la 1ère extraction par le Triton X-114 AFTER 1st EXTRACTION BY TRITON X-114  
 Extr. 2 Après la 2ème extraction par le Triton X-114 AFTER 2nd EXTRACTION BY TRITON X-114  
 Extr. 3 Après la 3ème extraction par le Triton X-114 AFTER 3rd EXTRACTION BY TRITON X-114  
 Final. Produit final après précipitation alcoolique FINAL PRODUCT AFTER ALCOHOL PRECIPITATION

(57) Abstract

The invention concerns a method for preparing a plasmid DNA using a wet cell biomass comprising after resuspension of said biomass the following steps: alkaline lysis, high ionic strength acidification, elimination of solubles, endotoxin and contaminating RNA reduction, filtering gel chromatography and conditioning. The invention also concerns a pharmaceutical composition containing a plasmid DNA and its use for gene therapy.

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## METHOD OF PREPARING PLASMID DNA

The subject of the present invention is a new protocol for purifying plasmid DNA which makes it possible to produce in a large quantity a DNA of acceptable pharmaceutical quality for use in humans. It also relates to a pharmaceutical composition comprising the DNA thus obtained and its use for the transfer of a nucleic acid into a host cell. The present invention is of particular interest for the purposes of gene therapy.

The transfer of genes into a given cell constitutes the very basis of gene therapy. This new technology, whose field of application is vast, makes it possible to envisage the treatment of serious diseases for which conventional therapeutic alternatives are not very effective or do not even exist and relates to both genetic diseases (hemophilia, cystic fibrosis, myopathy and the like) and acquired diseases (cancer, acquired immunodeficiency syndrome AIDS, and the like). The most widely used approach consists in using a viral vehicle to introduce the therapeutic nucleic acid into the cell to be treated and, in particular, a retroviral and adenoviral vehicle. Indeed, viruses have developed sophisticated mechanisms for crossing the cell membranes, for avoiding degradation at the level of the lysosomes and for causing their genome to penetrate into the nuclei in order to bring about the expression of the therapeutic gene. However, the viral approach has its limitations, in particular a limited cloning capacity, a potential production of replication-competent viral particles capable of spreading in the host organism and the environment, a risk of insertional mutagenesis in the case of retroviral vectors and, as regards adenoviral vectors, induction of immune and inflammatory responses in the host which hamper repetition of treatment. These major disadvantages in the context of a human use



justify the search for alternative systems for the transfer of nucleic acids.

5 An increasing number of methods for the transfer of genes make use of nonviral vectors. One of the most widely used consists in delivering the therapeutic nucleic acid by means of synthetic vectors such as cationic lipids which spontaneously interact with the nucleic acid to form positively charged complexes capable of fusing with the anionic cell  
10 membranes and of bringing about the penetration of the nucleic acid which they are transporting (see for example Behr, Bionconjugate Chemistry (1994) 5: 382). A so-called biolistic method ("gene gun") using bombardment of cells with DNA-coated metallic microprojectiles  
15 was recently used in the context of an anti-AIDS trial (Woffendin et al., 1996, Proc. Natl. Acad. Sci. USA 93, 2889-2894). Finally, an even simpler approach may also be envisaged by direct administration of naked DNA, especially in the context of diseases affecting the  
20 muscles, by intramuscular injection. These nonviral methods generally use a plasmid vector carrying the therapeutic gene and the elements necessary for its expression.

The carrying out of clinical trials based on  
25 nonviral methods requires being able to produce large quantities of plasmid DNA of pharmaceutical quality. The methods conventionally used are not optimal since they use enzymes of animal origin (lysozyme, proteinase, ribonuclease and the like), organic solvents known  
30 for their toxicity (phenol, chloroform) and mutagenic compounds (ethidium bromide) which are likely to contaminate the final product. Furthermore, their use on an industrial scale is difficult to carry out.

International applications WO95/21250 and  
35 WO96/02658 describe methods of preparing plasmid DNA in purified form, which methods can be used for human trials. However, it is known that a number of variables can influence the efficiency of preparative methods, especially the plasmid to be purified, its size, the



microorganism producing it, the fermentation medium and conditions. In this context, it is advantageous to be able to have a new method for the production of large quantities of plasmid DNA of pharmaceutical quality.

5 A new method of preparing plasmid DNA has now been found which comprises a succession of steps which are easy to carry out, avoiding the use of products of animal origin, toxic products and mutagenic products such as those cited above and which can be adapted to  
10 an industrial scale. The DNA is produced with a high yield, in a substantially pure form and of a quality which is compatible with a human use. The residual contaminations with proteins, endotoxins, RNA and genomic DNA of the producing microorganism are particu-  
15 larly low or even nondetectable by standard detection techniques. The examples which follow also show that this method allows the purification, in an efficient manner, of a large-sized plasmid incorporating the cDNA for dystrophin, intended for the treatment of  
20 Duchenne's myopathy.

Accordingly, the subject of the present invention is a method for preparing a plasmid DNA from a wet cell biomass harvested after fermentation of a producing cell comprising said plasmid DNA,  
25 characterized in that it comprises the following steps:

- a) alkaline lysis of the resuspended biomass, after resuspension of the wet cell biomass
- b) acidification at a high ionic strength,
- 30 c) removal of the insoluble matter,
- d) reduction of the endotoxins and of the ribonucleic acids (RNA),
- e) gel filtration chromatography, and
- f) conditioning.

35

For the purposes of the present invention, "plasmid DNA" designates an extrachromosomal cellular element consisting of a generally circular DNA molecule capable of autonomous replication in a producing cell



(the cell in which it is amplified). The choice of plasmids which can be used in the method of the present invention is vast. They may be of any origin (prokaryotic or eukaryotic) or may be formed by the assembly of a variety of elements. In general, plasmids are known to persons skilled in the art. A large number of them are commercially available but it is also possible to construct them by genetic engineering techniques (Maniatis et al., 1989, Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). There may be a cloning or expression vector derived for example from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogen) or alternatively pPoly (Lathe et al., 1987, Gene 57, 193-201).

Advantageously, a plasmid used in the context of the present invention possesses the genetic elements which allow it to replicate autonomously in the producing cell and, optionally, in a host cell (cell in which the therapeutic effect is sought). Such elements may consist, inter alia, of a replication origin allowing initiation of replication in a bacterium, a yeast, a fungus or a mammalian cell. It may be isolated from a prokaryot (ColE1 and the like), from a eukaryot (2 $\mu$  or ARS for autonomously replicating sequence), from a virus (SV40 ori from the simian virus 40, oriP from the Epstein-Barr virus EBV and the like) or from a bacteriophage (flori and the like). The choice of the appropriate replication origin is within the capability of persons skilled in the art. For example, for a plasmid intended to be produced in the microorganism *Escherichia coli* (*E. coli*), the ColE1 origin will be selected. Furthermore, if it is desired that it should be self-replicating in a mammalian host cell, it will also comprise an origin which is functional in a eukaryot, for example oriP, and it may also include the gene encoding the EBNA-1 protein of the EBV virus which is necessary for replication using it (Lupton and



Levine, 1985, Mol. Cell. Biol. 5,2533-2545; Yates et al., Nature 313, 812-815).

Moreover, a plasmid in use in the present invention may, in addition, comprise a selectable gene  
5 which makes it possible to select or identify the cells transfected (producing cells and/or host cells). It is possible to apply the methods of selection based on the principle of deficient producing cells (through auxotrophic mutations or introduction of a lethal gene)  
10 which are incapable of growing in the absence of a plasmid carrying a gene complementing this deficiency (for example dap system described in Patent EP 0,258,118, complementation of an auxotrophic mutation, use of genes encoding a suppressor tRNA supE,  
15 supF and the like). Another practice which is commonly used consists in integrating into the plasmid a gene encoding resistance to an antibiotic (ampicillin, kanamycin, tetracycline and the like). Of course, it may comprise additional elements which enhance its  
20 retention and/or its stability in a host or producing cell. In this regard, there may be mentioned the cer sequence whose presence promotes the monomeric retention of a plasmid (Summers and Sherrat, 1984, Cell 36, 1097-1103) and some sequences of viral origin  
25 (retrovirus LTR, ITR from an adeno-associated virus and the like) or of cellular origin allowing integration into the chromosomes of the host cell.

In accordance with the aims pursued by the present invention, a plasmid in use in the present  
30 invention is intended to transport one or more genes of therapeutic interest into a host cell. In general, the gene of interest may encode an antisense RNA, a messenger RNA which will then be translated into a polypeptide of interest, a ribozyme or alternatively an  
35 RNA which confers a direct therapeutic benefit (VA RNA of an adenovirus capable of repressing the immune response, RNA which activates the synthesis of interferon) (Abbas et al., in Cellular and Molecular



Immunology; W.B., Saunders Company Harcourt Brace Jovanovich Inc. p. 228).

5 The gene of interest may be isolated by any conventional technique such as cloning, PCR (Polymerase Chain Reaction) or alternatively synthesized chemically. It may be of the genomic type (equipped with one or more introns) or complementary DNA (cDNA). The polypeptide of interest may consist of a mature protein, a precursor and in particular a precursor  
10 intended to be secreted and comprising a signal peptide, a truncated protein, a chimeric protein derived from the fusion of sequences of various origins or alternatively a mutated protein having improved and/or modified biological properties.

15 By way of examples, use may be made of a gene of interest selected from those encoding the following polypeptides:

- 20 - cytokines or lymphokines ( $\alpha$ -,  $\beta$ - and  $\gamma$ -interferons, interleukins and especially IL-2, IL-6, IL-10 or IL-12, tumor necrosis factors (TNF), colony-stimulating factors (GM-CSF, C-CSF, M-CSF and the like);
- 25 - cellular or nuclear receptors, especially those recognized by pathogenic organisms (viruses, bacteria or parasites) and, preferably, by the HIV virus (Human Immunodeficiency Virus) or their ligands;
- 30 - proteins involved in a genetic disease (factor VII, factor VIII, factor IX, dystrophin or minidystrophin, insulin, CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein, growth hormones (hGH);
- 35 - enzymes (urease, renin, thrombin and the like);
- enzyme inhibitors ( $\alpha$ 1-antitrypsin, antithrombin III, viral protease inhibitors and the like);
- polypeptides with antitumor effect which are capable of inhibiting, at least partially, the initiation or the progression of tumors or cancers



- (antisense RNA, antibodies, inhibitors acting on cell division or transduction signals, products of expression of tumor suppressor genes, for example p53 or Rb, proteins stimulating the immune system and the like);
- proteins of the major histocompatibility complex classes I or II or regulatory proteins acting on the expression of the corresponding genes;
  - polypeptides capable of inhibiting a viral, bacterial or parasitic infection or its development (antigenic polypeptides having immunogenic properties, antigenic epitopes, antibodies, transdominant variants capable of inhibiting the action of a native protein by competition and the like);
  - toxins (thymidine kinase of the herpes simplex virus 1 (TK-HSV-1), ricin, cholera or diphtheria toxin and the like) or immunotoxins; and
  - markers ( $\beta$ -galactosidase, luciferase and the like).

Of course, the gene of interest may be placed under the control of elements necessary for its expression in the host cell. "Elements necessary for its expression" designate all the elements allowing its transcription into RNA and the translation of an mRNA into a polypeptide. Among these, the promoter is of particular importance. It may be derived from any gene (eukaryotic, viral, natural promoter of the gene of interest in question and the like) or it may be artificial. Moreover, it may be constitutive or regulatable. Alternatively, it may be modified so as to enhance the promoter activity, suppress a region inhibiting transcription, modify its mode of regulation, introduce a restriction site and the like. There may be mentioned, by way of examples, the CMV (Cytomegalovirus) viral promoter, RSV (Rous Sarcoma Virus) viral promoter, HSV-1 virus TK gene promoter, SV40 virus early promoter, MLP adenoviral promoter and





the like, or alternatively eukaryotic promoters of the murine or human PGK (phospho glycerate kinase) gene,  $\alpha$ 1-antitrypsin gene (liver-specific), and immunoglobulin genes (lymphocyte-specific).

5           Such elements may also comprise additional elements such as introns, signal sequence, nuclear localization sequence, sequence for termination of transcription (polyA), site for initiation of translation of the IRES type or of another type, and the like.

10           A plasmid in use in the present invention is amplified in a producing cell before being purified according to the method of the present invention. Gram-negative bacteria and in particular *E. coli* are most particularly preferred. As a guide, there may be  
15 mentioned the strains DH5 (Grant et al., 1990, Proc. Natl. Acad. Sci. USA 87, 4645-4649), MC1061 (Wertman et al., 1986, Gene 49, 253-262) and its derivatives such as DH10B (Grant et al., 1990, *supra*). Given that this  
20 is a technology which is widely known to date, only a brief description will be given of the procedure to be carried out in order to introduce a plasmid into a bacterium and to amplify it. All conventional techniques may be used in the context of the present invention (treatment with calcium, rubidium and  
25 hexaminecobalt chlorides, with reducing agents, with DMSO, electroporation, transduction, liposomes and the like; Maniatis et al., 1989, *supra*). The producing cells thus transformed are then altered according to general practices in the art (continuous, "batch" or  
30 "fed batch" fermentation. The culture conditions can be easily established by persons skilled in the art on the basis of the general knowledge in this field and of the selection system carried by the plasmid. Their harvest is carried out according to the usual techniques, such as  
35 as filtration or alternatively low-speed centrifugation, in order to generate the wet cell biomass which can, at this stage, be frozen or stored at 4°C before being subjected to the method according to the invention.



According to the method of the invention, the lysis of the wet cell biomass is carried out after having carried out its resuspension. A resuspension buffer is generally used which is slightly basic in order to neutralize the acidic character of the cellular paste and which is of a low ionic strength having little or no lytic effect on the transformed cells. Its composition and its pH can vary in particular according to the producing cell, the culture medium used or any other parameter. Persons skilled in the art are capable of preparing an appropriate resuspension buffer. There may be mentioned, by way of example, a buffer containing EDTA (concentration of 1 to 50 mM, preferably 10 mM) and Tris-HCl (concentration of 10 to 100 mM, preferably 50 mM) buffered to a pH of about 8. The cells can be resuspended by any usual technical means such as rectilinear stirring, repeated pipetting and/or homogenizer (vortex, homogenizer via shearing and the like).

The alkaline lysis step makes it possible to release the cellular content and to solubilize all the components thereof. Proteins, RNA and DNA are denatured including the plasmid DNA whose two homologous strands remain entangled, unlike the genomic DNA. It may be advantageous to carry out the alkaline lysis in the presence of a detergent and, preferably, of an anionic surfactant. The choice of the base and of the surfactant is not limited. In this regard, the combination sodium hydroxide and SDS (sodium dodecyl sulfate) is preferred, especially at final concentrations of about 0.1 M and 0.5% respectively. The final pH of the lysis solution is preferably between 11 and 13 and, optimally, between 12.2 and 12.4. It is indicated that it is preferable to mix the resuspended transformed cells and the lysis solution gently, for example by inverting, so as to minimize breaks in the genomic cellular DNA which would then be capable of contaminating the plasmid DNA preparation. Although this is not a preferred embodiment, it is nevertheless possible, in



the context of the present invention, to facilitate the cell lysis by heating at a high temperature (see for example international application WO96/02658) or the use of animal enzymes which degrade the cell membranes (lysozyme and the like).

The second step of the method according to the invention results in acidification, at high ionic strength, of the lysate obtained above. The acidification is preferably carried out suddenly, that is to say in a single step. Under these conditions, the plasmid DNA is rapidly renatured while the great majority of proteins, of denatured genomic DNA and of the RNA species which are insoluble under a high ionic strength condition circulate. In the context of the present invention, a solution comprising a buffer or a strong acid combined with a salt whose pH is between 4.5 and 6.5 is used. According to an advantageous embodiment, a solution of potassium acetate at a final concentration of close to 1 M is preferably used so as to obtain a final pH of about 5.1. However, it would also have been possible to use a solution of sodium acetate having a pH and a concentration as indicated above.

The insoluble matter consisting of cellular debris and flocculates of macromolecules are then removed. This may be carried out by any conventional filtration or centrifugation technique. It may be judicious to remove most of the insoluble matter first by centrifugation and then to continue the clarification by filtration. Many filters can be used in the context of the present invention provided that they retain the insoluble matter and allow the plasmid DNA to pass through. Advantageously, the filter chosen will have a porosity of between 1 and 100  $\mu\text{m}$ , more advantageously 2 and 75  $\mu\text{m}$ , preferably 5 and 75  $\mu\text{m}$ , most advantageously 3 and 50  $\mu\text{m}$ , and most preferably 10 and 50  $\mu\text{m}$ . It may be made of a synthetic material such as nylon, an organic material such as cellulose or a nonorganic material such as glass. According to an advantageous embodiment, successive filtrations are



carried out with the aid of filters of decreasing porosities, for example a first filtration on sintered glass having a porosity of between 100 and 40  $\mu\text{m}$  (No. 2 sintered glass, Schott AG), the second on sintered glass having a porosity of 16 to 40  $\mu\text{m}$  (No. 3 sintered glass, Schott AG) and the last on sintered glass having a porosity of 10 to 16  $\mu\text{m}$  (No. 4 sintered glass, Schott AG).

According to another variant, it is possible to carry out a single filtration using polypropylene cartridge Sartopure PP having a porosity of 8  $\mu\text{m}$  (Ref. 552 1302 P9-00-A) or Sartopure PP2 having a porosity of 3  $\mu\text{m}$  (Ref. 559 1302 P9-00-A).

According to an optional but particularly advantageous embodiment, the filtrate may be concentrated before the next step of reduction of endotoxins. The means for concentrating a DNA dissolved in an aqueous solution are known to persons skilled in the art. There may be mentioned ultrafiltration, alcohol precipitation or alternatively a combination of these two techniques.

As regards ultrafiltration, various membranes can be used as long as they do not or only slightly adsorb the plasmid DNA under the conditions for use. Membranes may be advantageously used whose cut-off is between 20 and 300 kDa, preferably between 30 and 100 kDa. They may be of varied compositions, organic or otherwise (poly(ether)sulfone, cellulose acetate and the like). The membranes which are particularly suitable are those of the YM type (and in particular YM30-76, Diaflo and YM30-4208, Centricon), those with which the Easy Flow units are equipped (reference 14669-OS-1V or 14669-OS-2V, Sartorius) or alternatively minipellicon 2 PL300 (Millipore made of regenerated cellulose). Ultrafiltration constitutes, at this step, a powerful means of reducing the contamination of the plasmid DNA preparation with pigments of cellular origin or derived from the culture medium.



It is also possible to concentrate the nucleic acids by alcohol precipitation in the presence of ethanol or isopropanol. The precipitation parameters such as volume of alcohol to be added, temperature, 5 presence of monovalent cations as well as the recovery of the precipitated material are detailed in many manuals accessible to persons skilled in the art. In particular, precipitation with isopropanol has the advantage of further reducing the content of pigments, 10 some of them being soluble in the alcoholic phase.

According to a preferred mode, the filtrate is first concentrated by ultrafiltration on a polysulfone membrane having a cut-off of about 100 kDa with the aid of a disposable Easy Flow type unit (Sartorius) or on a 15 minipellicon 2 PL300 membrane (Millipore) having a cut-off of about 300 kDa. Once the volume is reduced by a factor of 5 to 20, the nucleic acids are precipitated by addition of 0.7 volume of isopropanol. The precipitated material is recovered by centrifugation and may 20 be subjected to one or more washes in ethanol at a concentration of 70 to 80% in order to reduce the alcohol-soluble contaminants as salts and, as already mentioned, residual pigments. After drying, the nucleic acids are taken up in an appropriate buffer, for 25 example 10 mM Tris-HCl, pH 8, containing EDTA at a concentration of about 1 mM, in order to inhibit the nucleases, and optionally sodium acetate (at a final concentration of about 0.3 M which allows the precipitation of the nucleic acids after the step of 30 extraction with Triton). It is important to note here that the ultrafiltration and isopropanol precipitation steps are particularly advantageous in order to remove most of the pigments from the preparation.

At this stage, the plasmid DNA preparation 35 contains large quantities of RNAs and of endotoxins and the following steps consist in a reduction of their levels. Reduction is understood to mean a substantial reduction in the level of endotoxins or RNA between the beginning and the end of the step, by a factor of at



least 100 and, preferably, of at least 1000. The concentration of RNA and endotoxins may be assessed by tests similar to those described below or by any other methodology disclosed in the literature. Although the steps can be interchanged, it is preferable, in the first place, to act on the endotoxins and then on the RNAs.

Endotoxins, because of their pyrogenic character, should be considerably reduced or even eliminated before envisaging administration to humans. As regards common pharmaceutical products, the maximum dose which can be tolerated has been set by the health authorities at 5 units (EU) per dose. Now, it has been shown that common methods of preparing plasmid DNA (ultracentrifugation on cesium chloride gradient, anion-exchange chromatography and the like) allow large quantities of endotoxins to remain (Cotten et al., 1994, Gene Therapy 1, 239-246).

For the purposes of the invention, use will be preferably made of an extraction in the presence of a nonionic detergent having a cloud point of between 15°C and 35°C, advantageously 18°C and 30°C and, preferably, 20°C and 25°C. A preferred detergent is chosen from the polyoxyethylenes. Examples of detergents which can be used according to the present invention are described in the following table:



Detergent	Cloud point (°C)	Density (20°C)	Formula	Supplier
Brij58	45°C	-	$C_{16}H_{33}(OCH_2CH_2)_{20}OH$	ICI Americas
Triton™ X-114	22°C	1.054	$(CH_3)_3C-CH_2-C(CH_3)_2-C_6H_4-(OCH_2CH_2)_{7-8}OH$	Sigma
Tergitol™ TMN6	37°C	1.009	$C_{12}H_{25}(OCH_2CH_2)_8OH$	Sigma
Tergitol™ NP7	41°C	1.048	$C_9H_{19}-C_6H_4-(OCH_2CH_2)_{7-8}OH$	Sigma
Tergitol™ Min-Foam IX	40°C	0.995	$C_{11-15}H_{23-31}-O(CH_2CH_2O)_x [CH_2CH_2O/CH_2CH(CH_3)O]_yCH_2CH(CH_3)OH$	Sigma
Tergitol™ Min-Foam 2X	20°C	0.978	$C_{11-15}H_{23-31}-O(CH_2CH_2O)_x [CH_2CH_2O/CH_2CH(CH_3)O]_yCH_2CH(CH_3)OH$	Sigma

The compounds described above are amphiphilic compounds whose miscibility in the aqueous phase may be controlled by varying the temperature around their cloud point. Advantageously, according to the method of the invention, the DNA preparation is cooled to a temperature of less than 10°C before adding said detergent. The final concentration of detergent to be used may be between 0.5 and 6%, advantageously between 1 and 5% and, most preferably, at around 1%. Under these conditions, said detergent is soluble in water and forms micelles which complex the endotoxins. After incubation and centrifugation of the plasmid DNA/detergent mixture at a temperature considerably greater than the cloud point (for example >37°C in the case of Triton™ X-114), there is separation of two phases: an aqueous phase containing the plasmid DNA and a phase containing the detergent and the endotoxins. When the detergent chosen has a density greater than that of the DNA solution, after separation of the phases by centrifugation, the aqueous phase is located



in the top part of the tube and the phase containing the detergent and the endotoxins in the bottom part, and conversely when the detergent has a density less than that of the DNA solution. For obvious technical reasons, a detergent will be preferably chosen which has a density greater than that of the DNA solution. Persons skilled in the art have, in addition, the necessary knowledge allowing them to adjust, if necessary, the density of the DNA solution by modifying, for example, the salt concentration of said solution. The method according to the invention may comprise one or more (preferably 3) successive extractions as described above.

According to the present invention, a preferred detergent consists of Triton<sup>TM</sup> X-114 (octoxynol or octylphenoxy-poly(ethyleneglycoether)<sub>n</sub> with n = 7 or 8) whose cloud point is about 20°C and the density about 1.06.

The present invention also relates to a variant of the method of the invention according to which the nonionic detergent chosen has a cloud point situated outside the recommended range, and according to which said cloud temperature is adjusted by the addition of a small quantity of an anionic detergent ("Nonionic Surfactants", Chapter "Surfactant and Deterative Systems" in Kirk-Othmer Encyclopaedia of Chemistry, John WILEY & Sons, 1995).

According to an advantageous variant of the method according to the invention, the reduction in the endotoxins is followed by a step of alcohol precipitation of the plasmid DNA by incubating at cold temperature (4°C, -20°C or -80°C) in the presence of 0.3 M sodium acetate and about 70% ethanol. The precipitate of nucleic acid is conventionally recovered by centrifugation. It may be washed with an 80% ethanol solution in water before being dried and redissolved in an aqueous medium such as for example 10 mM Tris-HCl, pH 8, 1 mM EDTA. This precipitation step, which is





moreover optional, offers an effective means of removing traces of residual Triton<sup>TM</sup> X-114.

The reduction in the contamination by RNAs may be carried out by any means known in the art, for example enzymatic hydrolysis by means of a ribonuclease of animal origin such as bovine pancreatic ribonuclease A. However, in the context of the present invention, it is preferable to use a selective precipitation of the RNAs under conditions of high ionic strength and in the presence of dehydrating agents. Various salts may be used and there may be mentioned, as a guide, lithium chloride (Ze'ev Lev, 1987, Analytical Biochemistry 160, 332-336), calcium chloride, ammonium acetate and ammonium sulfate. In this regard, ammonium sulfate constitutes a preferred embodiment, particularly at a final concentration of between 1 and 3.5 M, preferably between 1.5 and 3 M and, most preferably, between 2 and 2.5 M. According to another variant of the invention, calcium chloride may be used at a final concentration of between 10 mM and 2 M, advantageously between 20 mM and 0.5 M, and preferably between 50 mM and 0.1 M. Optimally, after the addition of the salt or of the saline solution, the mixture is kept gently stirred, optionally at low temperature, for a variable period (1 to 120 min), centrifuged and the plasmid DNA recovered in the supernatant.

The method according to the invention comprises, at this stage, a step of exclusion chromatography on gel filtration supports, which makes it possible to complete the purification of the plasmid DNA preparation (reduction of the residual RNAs and proteins) and also to bring about desalting. The choice of supports is broad and within the capability of persons skilled in the art. Supports approved for human or veterinary use by the competent American authorities (FDA for Food and Drug Administration) and/or the European Union agencies and having a high exclusion limit, in particular greater than or equal to



20 x 10<sup>6</sup> Da (as measured on polymers such as dextrans) will be more particularly selected. There may be mentioned, for example, the supports Sephacryl S500 HR (Pharmacia, reference 17-0613-01), S1000 SF (Pharmacia, reference 17-0476-01) and GF2000 (Biosepra, reference 260651). The Sephacryl S500 support is preferred in the context of the invention.

The column is initially equilibrated under saline conditions which limit the hydrophobic interactions between the support and the DNA. Advantageously, TEN buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA and 100 mM NaCl) is used. The chromatography conditions may be adjusted as a function of various parameters and in particular the column volume, the support chosen, the plasmid DNA concentration in the preparation and the size of the plasmid DNA. The plasmid DNA is excluded from the phase and is eluted before the low-molecular weight contaminants. The fractions containing it may be analyzed by the customary techniques (absorbent at 254 nm, visual analysis after separation by agarose gel electrophoresis and the like). It is also possible to connect the column to a detector provided with a filter (at 254 nm for example) for the on-line detection of positive fractions. It will be noted that one advantage of the method according to the invention consists in the removal, during this step, of the residual salt derived from the preceding step.

According to an optional embodiment, the fractions obtained after the chromatographic step may be combined and concentrated according to the methodology indicated above (ultrafiltration and/or alcohol precipitation).

Finally, the method according to the invention comprises a step for conditioning the plasmid DNA preparation. The conditioning buffers which can be used in the context of the present invention are varied. There may be a physiological saline solution (0.9% NaCl), a Hepes-Ringer, Lactate-Ringer or TE (10 mM Tris-HCl, pH 7.5 to 8, 1 mM EDTA) solution or simply



H<sub>2</sub>O. Optionally, the preparation may be subjected to a sterilizing filtration. Use will be advantageously made of 0.22 µm filters having a surface area which is suited to the volume to be treated. There may be mentioned, for example, the filtration units of the type including Minisart (Sartorius, reference SM16534), Sartolab P20 (Sartorius, reference 18053D), Millex GF (Millipore, reference SLGS025BS), Millex GV (Millipore, reference SLGV025BS), Millex GP (Millipore, reference SLGPR25LS), Anotop 25 (Whatman, reference 20025H-68092122), Anotop 25 Plus (Whatman, reference 2002AP-68094122), Sartobran 300 (reference 5231307H5p00V) or Easy Flow 0.2 µm made of cellulose acetate (reference 12307-05-1-V). Next, the filtrate is conditioned in doses adjusted to a given concentration.

The plasmid DNA concentration may be determined in a conventional manner, for example by spectrophotometry at a wavelength of 260 nm. The relative proportion of the different topoisomers may be visually evaluated by agarose gel electrophoresis and staining with ethidium bromide optionally followed by a densitometric analysis. The integrity of the plasmid may be checked by digestion with restriction enzymes having one or more cleavage sites.

The quality of the plasmid DNA prepared by the method according to the invention may be assessed by standard assays such as those which are described in the examples which follow. Contamination with RNA may be evaluated visually by agarose gel electrophoresis and staining with ethidium bromide or by spectrophotometry after hydrochloric orcinol reaction (Bial reagent) (Moulé, 1953, Arch. Science Physiol. 7, 161; Mejbaum, 1939, Hope Seyler Z. 258, 117). A plasmid DNA purified according to the method of the invention preferably has a residual RNA concentration of less than 5% (mass/mass), advantageously less than 3%, preferably less than 2% and most preferably less than 1%.



The residual contamination with proteins may be measured by any technique for assaying proteins exhibiting little or no interference caused by the DNA. An appropriate technique is that of the BCA (bicinchoninic acid) technique based on the spectrophotometric detection at a wavelength of 562 nm of the colored complex formed between BCA and the  $\text{Cu}^+$  ions derived from the reduction, in an alkaline medium, of cuprous ions  $\text{Cu}^{++}$  by proteins (Smith et al., 1985, Anal. Biochem. 150, 76-85). A plasmid DNA purified according to the method of the invention preferably has a residual protein contamination of less than 3% (mass/mass), advantageously less than 2%, preferably less than 1% and most preferably less than 0.5%.

The techniques for assaying endotoxins are known to persons skilled in the art. It is possible, for example, to carry out a colorimetric assay derived from the LAL (Limulus Amebocyte Lysate) method recommended by the European Union and United States pharmacopoeias, as used in commercial kits (Bio-Whittaker, QCL-1000, reference L50-647-U; Biogenic, COATEST, reference 82 2387). Preferably, the quantity of endotoxins in the plasmid DNA preparation is less than 50 EU, advantageously less than 20 EU, preferably less than 10 EU and most preferably less than 5 EU per mg of plasmid.

The contaminating chromosomal DNA may be assayed by the competitive quantitative PCR technique based on the amplification of sequences specific to the producing microorganism, by Southern or alternatively by "Slot-blot" with the aid of a specific probe. A plasmid DNA purified according to the method of the invention preferably has a residual chromosomal DNA contamination of less than 5% (mass/mass), advantageously less than 3%, preferably less than 2% and most preferably less than 1%.

A method according to the invention is particularly advantageous as regards the preparation of plasmid DNA having a size greater than 10 kb.



The subject of the present invention is also a pharmaceutical composition comprising a plasmid DNA purified by the method according to the invention as therapeutic or prophylactic agents. A pharmaceutical  
5 composition according to the invention may be used in various types of host cells. They are preferably a mammalian cell and in particular a human cell. Said cell may be a primary or tumor cell of hematopoietic origin (totipotent stem cell, leukocyte, lymphocyte,  
10 monocyte, macrophage and the like), of hepatic, epithelial or fibroblast origin and most particularly a muscle cell (myoblast, myocyte, satellite cell, cardiomyocyte and the like), a tracheal or pulmonary cell. Moreover, a composition according to the  
15 invention may comprise an element for targeting towards a particular cell, for example a ligand for a cellular receptor or alternatively an antibody. Such targeting elements are known.

A composition according to the invention may be  
20 administered by the systemic route or by aerosol, in particular by the intragastric, subcutaneous, intracardiac, intramuscular, intravenous, intraperitoneal, intratumor, intrapulmonary, intranasal or intratracheal route. The administration may be made in a single dose  
25 or in a dose repeated once or several times after a certain interval of time. The appropriate route of administration and dosage vary according to various parameters, for example the individual or the disease to be treated or alternatively the gene(s) of interest  
30 to be transferred. In particular, a pharmaceutical composition according to the invention may be formulated in the form of doses comprising between 0.05 and 100 mg of plasmid DNA purified according to the method according to the invention, advantageously 0.1  
35 and 10 mg and preferably 0.5 and 5 mg. The formulation may also include other compounds such as a pharmaceutically acceptable adjuvant or excipient.

In this context, it may be particularly advantageous to combine the plasmid DNA with a compound



which enhances its diffusion, especially a polymer or a cationic lipid. By way of examples, there may be mentioned 5-carboxyspermylglycin dioctadecylamide (DOGS),  $3\beta$ [N-(N',N'-dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol), (2,3-dioleoylocyl-N-[2-(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium tri-fluoroacetate) (DOSPA), spermine cholesterol and spermidine cholesterol (which are described in French application 96 01347).

Moreover, such a composition may, in addition, comprise an adjuvant capable of enhancing its transfecting power. It may be preferably a neutral lipid such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol and, in particular, dioleylphosphatidylethanolamine (DOPE). It is also possible to combine with the plasmid DNA/lipid complex other substances for further enhancing the transfection efficiency or the stability of the complexes.

A composition according to the invention is in particular intended for the preventive or curative treatment of diseases such as genetic diseases (hemophilia, cystic fibrosis, diabetes or Duchenne's or Becker's myopathies, and the like), cancers, viral diseases (hepatitis, AIDS and the like), and recurrent diseases (infections caused by the herpesvirus, the human papillomavirus and the like).

Finally, the present invention relates to the therapeutic or prophylactic use of a pharmaceutical composition according to the invention for the preparation of a medicament intended for the treatment of the human or animal body and, preferably, by gene therapy. According to a first possibility, the medicament may be administered directly in vivo (for example by intravenous or intramuscular injection, into an accessible tumor, into the lungs using an aerosol, and the like). It is also possible to adopt the ex vivo approach which consists in collecting cells from the patient (stem cells from the bone marrow, peripheral



blood lymphocytes, muscle cells and the like), transfecting them *in vitro* according to techniques of the art and then readministering them to the patient.

5 The invention also extends to a method of treatment using a plasmid DNA obtained by a method according to the invention, according to which a therapeutically effective quantity of the plasmid DNA is administered to a patient requiring such a treatment.

10 The present invention is more fully described with reference to the following figures:

15 Figure 1 illustrates a chromatogram after gel filtration on Sephacryl S500 (70 ml column, 16 mm in diameter and 350 mm in length) and loading of a 2 ml sample containing 5 mg of pCH110N obtained after alkaline lysis, ultrafiltration and treatment with ammonium sulfate. The elution is carried out at 0.5 ml/min (15 cm/h) in a 10 mM Tris-HCl buffer, 1 mM EDTA, 100 mM NaCl, pH 8.0. The optical density is  
20 recorded at 254 nm.

Figure 2 is a schematic representation of the vector pTG11025 containing the gene conferring resistance to kanamycin (kana), the replication origin of ColE1, the cytomegalovirus CMV promoter (pCMv), the  
25 intron of the gene encoding Hydroxy-Methylglutaryl-Coenzyme A Reductase (HMG), the cDNA encoding dystrophin and a sequence for polyadenylation of the RNAs transcribed (pA).

30 Figure 3 illustrates a chromatogram after gel filtration on Sephacryl S500 (5-liter column 8.5 cm in diameter and 82 cm in length) and loading of 125 ml sample containing 85 mg of pTG11025. The elution is carried out at about 15 ml/min (14.5 cm/h) in a 10 mM Tris-HCl buffer, 1 mM EDTA, 100 mM NaCl, pH 8.0.

35 Figure 4 illustrates the gradual removal of the endotoxins from an alkaline lysate by 3 successive extractions with 1% or 3% Triton<sup>TM</sup> X-114 followed by ethanol precipitation.



Figure 5 illustrates the selective precipitation of the contaminating RNAs of an alkaline lysate in the presence of increasing molarities of ammonium sulfate (0 to 3.2 M final).

5 Figure 6 illustrates the selective precipitation of the contaminating RNAs of an alkaline lysate in the presence of increasing molarities of calcium chloride (10 to 100 mM). The final molarity of  $\text{CaCl}_2$  is indicated under the relevant lines. S =  
10 deposition of a fraction of the sample of soluble material obtained after treatment with  $\text{CaCl}_2$ ; I. = deposition of a fraction of the sample of insoluble material obtained after treatment with  $\text{CaCl}_2$ ; oc =  
15 plasmid DNA in circular form (open circle); sc = plasmid DNA in supercoiled form (super coiled); a = RNAs.

The examples which follow illustrate only an embodiment of the present invention.

## 20 EXAMPLES

The solutions defined below were prepared from stock solutions or chemical products obtained commercially.

25

EXAMPLE 1:      Purification of the plasmid pCH110N from the transformed E. coli MC1061 strain

1. Preparation and amplification of the recombinant  
30 cells

35 Use is made of the E. coli MC1061 strain (Wertman et al., 1986, supra) and of the plasmid pCH110N. It is a plasmid of 8.5 kb whose retention in E. coli is brought about by a replication origin (ColE1) and a gene for resistance to ampicillin, both derived from pBR322. The gene of interest consists of the E. coli  $\beta$ -galactosidase reporter gene whose expression can be easily detected by staining with





X-Gal(4-chloro-5-bromo-3-indolyl- $\beta$ -D-glactopyranoside). It is provided in its 3' portion with a sequence encoding a eukaryotic nuclear localization signal. The nuclear localization of the recombinant  $\beta$ -galactosidase makes it possible to eliminate the problems of background noise generated by cross-reaction with the endogenous  $\beta$ -galactosidase of the host cell which is also detectable by Xgal, and therefore to bring about a specific detection of the enzymatic activity resulting from the transfixed plasmid. The expression of the reporter gene is directed by the SV40 early promoter.

The MC1061 cells are made competent by treating with calcium chloride and transformed with the plasmid pCH110N. The recombinant bacteria are selected on a selective medium. A clone is chosen by examining the restriction profiles from which a primary glycerol stock is constituted.

After inoculation of a preculture in a flask, it is used to inoculate a fermenter. The fermentation was carried out continuously (batch) in 18 liters of a 2 times concentrated LB medium (2xLB) without addition of a carbon substrate, at 37°C and in the presence of ampicillin (100  $\mu$ g/ml). The culture is collected after 2 hours in the stationary phase. Under these conditions and for a final optical density OD<sub>600</sub> of 7.5, 180 g of total biomass are obtained.

## 2 *Harvesting of the wet cell biomass*

The content of the fermenter is distributed into clean and sterile centrifuge bottles (Nalgene, reference 3122-1000, 3122-1010, 3120-1000 or 3120-1010) and the transformed cells recovered by low-speed centrifugation (5000 rpm (revolutions per minute) for 30 min) and at 4°C. It is possible to use a Sorvall RC3 centrifuge equipped with an H6000-A rotor having a capacity of 6x1 liter and to carry out three successive centrifugations in the same bottles in order to harvest the entire culture. After removal of the medium, the



weight of the cellular pellets is estimated by weighing and they can be frozen at  $-20^{\circ}\text{C}$  before being treated by the method detailed below. The cells thus harvested constitute the wet cell biomass.

5

### 3. Preparation of the acidified lysate

10 The frozen pellet is fragmented and the quantity of biomass which it is desired to treat is collected with the aid of a spatula. In the text which follows, the volumes of the solutions used are given per 27 g of wet biomass. The cells are taken up in 320 ml of resuspension buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8) previously equilibrated to  $4^{\circ}\text{C}$  and  
15 resuspended with the aid of a shearing homogenizer (Ultra Turrax-25 provided with a probe 18 mm in diameter) before being lysed in the presence of 320 ml of lysis buffer (1% SDS, 0.2 M NaOH) equilibrated to  $20^{\circ}\text{C}$ . The lysis is allowed to continue for 5 min at  
20 room temperature while gently stirring the preparation by inverting and then 320 ml of acid solution (3 M  $\text{CH}_3\text{COOK}$ , pH 5.5) are added equilibrated to  $4^{\circ}\text{C}$ . The acidified cell lysate is left for 20 min at  $0^{\circ}\text{C}$  while gently stirring regularly by inverting. The final pH is  
25 5.1.

### 4. Removal of the insoluble matter and concentration of the filtrate

30 The flocculate is first of all partially removed by low-speed centrifugation (5000 rpm for 30 min at  $4^{\circ}\text{C}$  in a GSA rotor, Sorvall). The supernatant is subjected to two successive filtrations on sintered glass of controlled porosity (16 to  $40\text{ }\mu\text{m}$  and then 10  
35 to  $16\text{ }\mu\text{m}$ ; sintered glass Nos. 3 and 4; Schott AG) with the aid of a suction flask connected to a water-jet pump or an equivalent source of vacuum.

The filtrate is subjected to a step of concentration by ultrafiltration on an Easy Flow



cartridge (Sartorius) equipped with a polysulfone membrane with a cut-off of 100 kDa (Sartorius, 0.1 m<sup>2</sup> reference 14669-OS-1-V or 0.2 m<sup>2</sup> reference 14669-OS-2-V depending on the volume to be treated). The cartridge  
5 is connected to a peristaltic pump and the recirculation flow rate applied is about 400 ml/min. The ultra-filtration is carried out until the final volume is reduced by a factor of 8 to 16. The work is carried out at room temperature in order to reduce the duration of  
10 the operation.

The nucleic acids contained in the filtrate are precipitated by addition of 0.7 volume of isopropanol kept at 20°C. The mixture is homogenized by successive inversions, incubated for 5 min at room temperature and  
15 the precipitate collected by centrifugation at 10,000 rpm for 30 min at 4°C (GSA rotor, Sorvall). After removal of the supernatant, the pellet of nucleic acids is washed twice in succession with 50 ml of an 80% ethanol solution in water (equilibrated to about  
20 -20°C) and again recovered by centrifugation at 10,000 rpm for 15 min at 4°C.

#### *Extraction of the endotoxins*

25 The pellet is dried and dissolved in 18 ml of a solution of sodium acetate (0.3 M CH<sub>3</sub>COONa in 10 mM Tris-HCl, 1 mM EDTA) and stored for about thirty minutes at 0°C. 2 ml of a solution of Triton<sup>TM</sup> X-114 (Sigma; reference X-114<sup>TM</sup>) at 10% (weight/volume) in  
30 0.3 M CH<sub>3</sub>COONa at pH 5.5 (1% final Triton concentration) are added and the mixture is homogenized by stirring manually. After incubating for 10 min on ice and then for 25 min at 52°C, the bottom phase obtained after centrifugation (SLA 1500 rotor, Sorvall)  
35 at 10,000 rpm for 10 min at 35°C is collected and eliminated. Two additional extractions are carried out under the same conditions in the presence of 2.2 ml and 2.4 ml of Triton<sup>TM</sup> X-114 respectively. The top phase is precipitated by addition of 2.5 volumes of absolute



ethanol at  $-20^{\circ}\text{C}$ . After incubating for at least 45 min at  $-20^{\circ}\text{C}$ , the precipitate is recovered by centrifugation at 10,000 rpm (SLA 1500 rotor, Sorvall) for 30 min and at  $4^{\circ}\text{C}$  and subjected to 1 or 2 successive washes with an 80% ethanol solution in water stored at  $-20^{\circ}\text{C}$ . The centrifugation pellet may be frozen before proceeding to the next step.

#### *Removal of the RNAs*

10

The centrifugation pellet is dried under vacuum and taken up in 9.4 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 8, at room temperature. Solid ammonium sulfate  $(\text{NH}_4)_2\text{SO}_4$  is added so as to have a final concentration of about 2 M. After mixing by inverting and incubating for 20 min on ice, the mixture is centrifuged for 30 min at 7000 rpm (SLA 1500 rotor, Sorvall) and at  $4^{\circ}\text{C}$ . A second centrifugation under identical conditions is carried out on the supernatant recovered from the first in order to complete the removal of the insoluble matter.

#### *Chromatography on Sephacryl S500*

25

The centrifugation supernatant is carefully collected and subjected to gel filtration chromatography on a Sephacryl S500 matrix (Pharmacia; reference 17-0613-01). Under the above-mentioned conditions, a column is used which has a capacity of 1 liter (length 622 mm, diameter 44 mm) developed at 3.8 ml/min (15 cm/h), but of course persons skilled in the art are capable of adjusting the capacity of the column depending on the volume to be treated.

35 The column is equilibrated in 2 volumes of TEN before applying a volume of nucleic acid sample representing 4 to 5% of the volume of the column. The collection and the detection of the fractions are automated (LKB 2212 fraction collector and LKB 2158 Uvicord SD detector equipped with a filter at 254 nm).



The fractions of 3 min are collected and frozen before being analyzed. Figure 1 illustrates a chromatogram obtained on a smaller scale but representative of the method. The plasmid DNA comes out in the exclusion  
5 volume whereas the RNA and proteins are retained and appear only later. The clear separation, with a return practically to the base line, obtained between the plasmid DNA peak eluted first and the RNA peak eluted second, may be noted.

10

#### *Conditioning*

The fractions containing the plasmid DNA are grouped together and concentrated about 8 x with the  
15 aid of an ultrafiltration unit of the Sartocn-Micro type with a polysulfone membrane having a cut-off of 100 kDa (Sartorius, reference 15669-00-1). Alternatively, and when a larger quantity of sample has to be treated, it is possible to use an EasyFlow  
20 ultrafiltration unit with a cellulose acetate membrane (20 kDa cut-off, Sartorius, reference 14549-OS-1V).

After concentration, the purified plasmid DNA sample is precipitated by addition of a solution of Na acetate (3 M, pH 5.5) to the final concentration of  
25 0.3 M and addition of 2.5 volumes of pure ethanol (99.95%) at -20°C. After incubating at -20°C (30 min), the plasmid DNA is recovered by centrifugation at 10,000 rpm for 30 min at 4°C (SLA 1500 rotor, Sorvall). The pellet is washed with 80% ethanol at about -20°C,  
30 and then dried and taken up in the appropriate conditioning buffer (TE, 0.9% NaCl, Hepes Ringer, Lactate Ringer, H<sub>2</sub>O; about 20 ml per aliquot of 27 g of cells treated).

After measuring the optical density at 260 nm,  
35 the DNA concentration is calculated by taking as base an OD<sub>260</sub> corresponding to 50 µg/ml. It can then be adjusted to 1.0 mg/ml by diluting with the conditioning buffer, and 20 mg of plasmid DNA are typically obtained per aliquot of 27 g of initial biomass.



EXAMPLE 2:      Purification of the plasmid pTG11025  
                         from the transformed *E. coli* DH10B  
                         strain

5

The *E. Coli* DH10B Electro Max strain is provided by Gibco BRL (Reference 18290-015). The plasmid pTG11025 (Figure 2) of 18.7 kb carries a marker gene which confers on the bacteria the capacity for  
10 resistance to kanamycin (gene encoding an aminoglycoside 3'-phosphotransferase which converts the antibiotic to an inactive derivative) and the ColE1 replication origin, these two elements ensuring the retention of the plasmid in the producing strain. It  
15 comprises, in addition, a cassette for expression of the cDNA encoding dystrophin under the control of the CMV promoter associated with the HMG intron.

The competent DH10B cells are transformed with the plasmid under the conditions recommended by the  
20 supplier and a primary glycerol stock is constituted which is stored at -80°C after selection of a recombinant clone. A primary seed batch is used to constitute a preculture in a flask on 2x LB medium in the presence of kanamycin 50 µg/ml. The culture is  
25 incubated at 30°C in a thermostatted stirrer (180 rpm) for 14 to 16 hours.

After transferring the preculture into an inoculation flask, the transformed strain is propagated in a 20-liter fermenter. The growth takes place at 30°C  
30 in a complex culture medium (Hycase SF 37.5 g/l, yeast extract 9 g/l supplemented with growth factors and inorganic salts) using glycerol as carbon substrate (20 g/l) and in the presence of kanamycin (50 µg/ml) for the selection pressure. The pH = 7.0 is regulated  
35 by automatic addition of sodium hydroxide (NaOH, 30%) and of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>, 2 M). Dissolved oxygen is maintained at a saturation greater than or equal to 25% (aeration rate of 1 v.v.m (10 l min<sup>-1</sup>) and a variable



stirring rate. It may be advantageous to add kanamycin during culture.

The culture is stopped by cooling at 4°C when the bacteria reach the stationary growth phase. The  
5 culture is drawn off and the biomass harvested by centrifugation (Sorvall RC3B centrifuge, 15 min, 4°C, 5000 rpm). The cell pellet is stored at -20°C until the method of purifying the plasmid pTG11025 is applied.

It is similar to the method described in  
10 Example 1, except for the following modifications:

- 15 - The purification is carried out using 360 g of wet biomass taken up in 3840 ml of resuspension buffer and lysed with an equivalent volume of lysis buffer and then of acid solution.
- The insoluble matter is removed by filtration on sintered glass No. 4 (16-10 µm, Schott AG) before concentrating 15 times on Easy Flow.
- 20 - The filtrate is separated into 6 aliquots. The precipitation with isopropanol, the washes with 80% ethanol, the extractions of the endotoxins with Triton™ X-114, the precipitation with ethanol and the subsequent washes with 80% ethanol are carried out on each of the aliquots  
25 as described in Example 1.
- The samples are grouped together for the precipitation with ammonium sulfate at a final molarity of 2 M.
- 30 - The centrifugation supernatant harvested after the ammonium sulfate precipitation step is loaded onto a Sephacryl-S500 gel filtration column having a volume of 5 liters (length 82 cm, diameter 8.9 cm) developed at a flow rate of 15 ml/min (14.5 cm/h). The chromatogram  
35 obtained (Figure 3) shows that the plasmid DNA is eluted in the exclusion volume, whereas the contaminating RNAs and other low-molecular-weight compounds are retained on the column.



- 5       - The fractions containing the purified plasmid DNA are grouped together and concentrated by ultrafiltration (factor 10.9). The plasmid DNA is then precipitated by addition of sodium acetate to a final concentration of 0.3 M and of 2.5 volumes of 99.95% ethanol at -20°C.
- 10       - The precipitate is collected by centrifugation (10,000 rpm at 4°C, Sorvall SLA 1500 rotor, 30 min) and washed with 200 ml of 80% ethanol.
- 15       - After drying under vacuum, the DNA is taken up in the TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) conditioning buffer, its concentration measured by UV spectrometry and adjusted to 1 mg/ml in the same buffer. 145 mg of purified plasmid DNA are typically obtained.

20       The integrity of the plasmid is evaluated by restriction enzyme mapping and the profiles obtained correspond to what is expected. Moreover, the presence of contaminants is also determined in the final preparation and the results presented below.

Contaminant	Method	Result
Proteins	BCA	0.49% (n=3)
RNA	Bial colorimetric reaction	2.48% (n=3)
Endotoxin	LAL colorimetric method	2.34 EU/mg (n=5)

25       Moreover, the functionality of the purified plasmid pTG11025 is checked by transfection of cell lines and detection of recombinant dystrophin by immunofluorescence.

30       Ten µg of purified plasmid DNA are combined with 40 µg of Lipofectin (a mixture of compounds facilitating the transfection of eukaryotic cells) following the instructions of the supplier (Life Technologies, Bethesda, USA, reference 18292-0011), and are then added, in 2 ml of DMEM medium (Life Technologies, Bethesda, USA, reference 11963), to

35





2.5 × 10<sup>6</sup> A 549 (human pulmonary adenocarcinoma) cells inoculated the day before in a dish 35 mm in diameter, cultured for 24 hours in the presence of DMEM supplemented with 10% (vol/vol) of fetal calf serum (Life Technologies, Bethesda, USA, reference 10101-061) and previously rinsed with a physiological buffer (1× PBS). Four hours after addition of the DNA, the medium is supplemented with 10% (vol/vol) of fetal calf serum and then the culture is continued for 48 hours. The cells are then fixed by treating at -20°C in a methanol/acetone (1/1) (vol/vol) mixture, air-dried, and incubated in the presence first of an anti-dystrophin monoclonal mouse antibody (Novocastra, Newcastle/Tyme, UK, reference NCL-DYS2) and then of an anti-mouse rabbit antibody (ICN, Costa Mesa, USA, reference 651713) coupled to FITC (fluorescein thioisocyanate); the detailed conditions for these operations are known to persons skilled in the art. The dystrophin produced during the expression of the gene encoded by the vector pTG11025 is detected by fluorescence microscopy examination of the immune complexes. The simultaneous examination of cells transfected in the presence of a control plasmid pTG11025 whose functionality has already been demonstrated, purified according to a standard protocol (Maniatis et al., 1989, *supra*) (positive control) or in the absence of DNA (negative control) makes it possible to evaluate the functional character of the plasmid DNA preparation pTG11025. The amount of fluorescent cells expressing recombinant dystrophin is comparable after transfection with the control pTG11025 and with the plasmid purified by the method according to the invention. Of course, the nontransfected cells show no fluorescence.

35



Tests of extractions with Triton™ X-114 were carried out on an alkaline lysate as obtained in Example 1 in order to determine the optimum concentrations of Triton™ X-114 to be used and the number of extractions to be carried out in order to reduce the level of endotoxins below the tolerated threshold ( $\leq 2$  EU/dose).

To do this, the initial alkaline lysate is divided into 2 batches subjected to 3 consecutive extractions in the presence of Triton™ X-114 at a final concentration of 1 and 3% respectively. The mixture is homogenized, incubated on ice (0°C) for a few minutes, centrifuged for 5 minutes (12,000 rpm, Eppendorf centrifuge, reference 5414) at room temperature. After centrifugation, the bottom phase (Triton™ X-114 and extracted endotoxins) of each batch is eliminated and the top aqueous phase (Extr. 1; nucleic acids and remaining endotoxins) again treated as described with 1% or 3% Triton™ X-114 after collection of one aliquot which serves for assaying the endotoxins (Extr. 2 and 3). After the third extraction, the top phase is precipitated by addition of 2.5 volumes of absolute ethanol. The precipitate is taken up in 10 mM Tris-HCl, 1 mM EDTA, pH 8 (final).

The level of endotoxins is assayed by a colorimetric test derived from the LAL method, with the aid of the Biogenic COATEST kit (reference 822387) and the results presented in Figure 4. A notable reduction in the level of endotoxins is observed after two extractions in the presence of 1% Triton. A similar result is obtained when 3% Triton™ X-114 is used. In the 2 cases, the endotoxin level measured in the final product is compatible with a pharmaceutical use for an average dose of 1 mg of plasmid DNA.



EXAMPLE 4:      Selective precipitation of the  
                         contaminating RNAs

5                    Selective precipitation tests were carried out  
on an alkaline lysate as obtained in Example 1 in order  
to determine the optimum ammonium sulfate concen-  
trations for precipitating the contaminating RNAs. To  
do this, the alkaline lysate is divided into 7 aliquots  
which are subjected to precipitation in the presence of  
10 an increasing quantity of ammonium sulfate (0.5 M, 1 M,  
1.5 M, 2 M, 2.5 M, 3 M and 3.2 M final).

                  The precipitated material recovered by  
centrifugation and the soluble material are analyzed by  
electrophoresis on a 0.4% agarose gel. After staining  
15 with ethidium bromide and visualizing by UV fluor-  
escence, the plasmid DNA appears in the form of sharp  
bands corresponding to the different topoisomers. In  
contrast, the RNAs form a very diffuse band which  
migrates in the bottom part of the gel.

20                  The results illustrated in Figure 5 show that  
above a final ammonium sulfate concentration of 1.5 M,  
the great majority of RNAs become selectively  
precipitated whereas the plasmid DNA remains soluble.  
The removal, in the precipitate, of large-sized nucleic  
25 acid spaces retained in the pockets of the analytical  
gel, should also be noted.

EXAMPLE 5:      Selective precipitation of the  
                         contaminating RNAs with calcium chloride

30

                  The selective precipitation of the RNAs was  
also tested in the presence of calcium chloride  
(CaCl<sub>2</sub>). An alkaline lysate prepared according to the  
method described in Example 1, including the step of  
35 treatment with Triton<sup>TM</sup> X-114, was aliquoted into  
identical samples to which various volumes of a  
concentrated CaCl<sub>2</sub> solution (1 M in this specific case,  
but a less concentrated solution, 0.5 M for example,  
can be used) are added in order to obtain a final



calcium chloride concentration of 10, 20, 30, 50, 75 or 100 mM, respectively. The soluble and insoluble fractions are separated by centrifugation, and the insoluble pellet is resuspended in a low-ionic strength buffer in the absence of  $\text{CaCl}_2$ . The soluble and insoluble samples are analyzed by agarose gel electrophoresis and ethidium bromide staining of a fraction of sample in order to separate the plasmid DNA (open circular and supercoiled forms) and the contaminating RNAs.

The results (Figure 6) show that above a  $\text{CaCl}_2$  concentration of 30 mM, a detectable precipitation of the RNAs is observed. This precipitation becomes substantial and then massive above 50 and 70 mM respectively and then practically complete at 100 mM. The plasmid DNA in its open circular (Open-Circle, migrating less rapidly) and supercoiled (Super-Coiled, migrating most rapidly) forms remains, for its part, predominantly soluble in each of the cases.

EXAMPLE 6:      Removal of the colored pigments

The efficiency of the removal of the colored pigments present after the alkaline lysis step (cf. Examples 1, 2 and 5) was evaluated during the steps of concentration by ultrafiltration, of precipitation with isopropanol and of washing with 80% ethanol.

The presence of these pigments results in a yellow color of the crude alkaline lysate before its concentration by ultrafiltration which can be analyzed by measuring the optical density at 340 nm. This absorbance was therefore measured on the samples obtained after the steps of initial ultrafiltration, of precipitation and of washing with isopropanol and with 80% ethanol. Two series of experiments were carried out, one involving filtration of the alkaline lysate on sintered glass, the other on polypropylene cartridge Sartopure PP. The initial lysates are obtained from 150 g of a cell biomass obtained from two fermentations



of the pair *E. coli* strain DH5 × plasmid pTG11025 (~19 kbp).

5 Experiment No. 1: Macrofiltration carried out on  
No. 3 sintered glass (16 to 40 µm  
of porosity - Schott AG

Step	Final volume (ml)	Optical density at 340 nm	Cumulative pigment yield	Cumulative removal factor (X)
Initial crude lysate	5340 ml	0.29	100%	1 X
Post macrofiltration	5260 ml	0.28	95.1%	1.1 X
Post concentration by ultrafiltration (Easy- Flow 300 kDa, Sartorius)	795 ml	0.38	19.5%	5.1 X
Post alcohol precipitation	432 ml	0.39	10.1%	9.2X

10 Experiment No. 2: Macrofiltration carried out on a  
cartridge Sartopure PP2 (porosity  
8 mm - Sartorius)

Step	Final volume (ml)	Optical density at 340 nm	Cumulative pigment yield	Cumulative removal factor (X)
Initial crude lysate	5200 ml	0.32	100%	1 X
Post macrofiltration	5000 ml	0.31	93.1%	1.1 X
Post concentration by ultrafiltration (Easy- Flow 300 kDa, Sartorius)	790 ml	0.57	27.0%	3.7 X
Post alcohol precipitation	432 ml	0.62	16.1%	6.2X

15 These results show that the steps of  
ultrafiltration and precipitation with isopropanol  
contribute to the removal of the pigments by a factor  
of 4 to 5 × and 1.5 to 2 ×, respectively. Combined,  
these two steps make it possible to achieve a removal



factor of 6 to 10 X, that is to say a total removal of 80 to 90% of the colored pigments.

5 EXAMPLE 7:      Purification of the plasmid pTG11025  
                         from the E. coli DH5 Library Efficiency  
                         strain

10        The E. coli DH5 Library Efficiency strain (Life Technologies; ref. 18262-014) is transformed with the plasmid pTG11025 (see Example 2 and Figure 2). The steps of transformation, preparation and harvesting of the biomass are identical to those described in Example 2.

15        The crude lysate is filtered on a filtration cartridge Sartopure PP2 (Sartorius, ref. 5591302P9-0) at a flow rate of 700 ml/min with the aid of a peristaltic pump. The filtrate is then concentrated on a Millipore Minipellicon II membrane made of regenerated cellulose of the PL300 type having a  
20        surface area of 0.1 m<sup>2</sup> (ref. P2C300C01) (recirculation flow rate ~400 ml/min, rate of drawing off between 60 and 30 ml/min so as to maintain a mean transmembrane pressure of less than or equal to  $0.5 \times 10^5$  Pa).

25        The nucleic acids thus concentrated (815 ml) are treated with isopropanol and with ethanol as described above in Example 2. The endotoxins are removed by three successive extractions with Triton<sup>TM</sup> X-114. The nucleic acids are precipitated. The residual salts and nonionic detergent are rinsed with the aid of  
30        aqueous ethanol.

35        The nucleic acids are taken up in a 10 mM Tris buffer, 1 mM EDTA, pH 7.5 and the RNAs are specifically precipitated in the presence of 100 mM CaCl<sub>2</sub> final. These RNAs are removed by centrifugation (10,000 rpm, Sorvall rotor, SLA 1500, temperature 20°C, 40 min). The plasmid DNA present in the soluble phase is precipitated by addition of isopropanol and resolubilized in a 10 mM Tris buffer, 1 mM EDTA, 250 mM NaCl, pH 8.



An aliquot of the sample corresponding to 180 g of initial biomass is deposited on a Sephacryl S500 column (volume of 6 l, length 92 cm, diameter 8.9 cm, flow rate of 5 ml/min or ~5 cm/hour. The absorbance of the eluate is monitored at 254 nm. The fractions containing the plasmid DNA are affected immediately after elution of the column dead volume (~2100 ml), whereas those containing the RNAs are harvested after about 2 dead volumes (~4100 ml).

The fractions containing the DNA are then grouped together (volume 1480 ml). The plasmid DNA is concentrated by ultrafiltration on an EasyFlow unit with a cellulose triacetate membrane with a cut-off of 20 kDa (Sartorius, ref. 14549-OS-1V). After precipitation with ethanol in the presence of sodium acetate, the plasmid DNA is washed with 80% ethanol and dried under vacuum and then taken up in a conditioning buffer 10 mM Tris, 1 mM EDTA, pH 7.5, and stored frozen at -20°C.

In order to analyze the quality of this DNA, the following parameters were measured:

Initial biomass:	360 g (wet weight) of pTG11025/DH5LE approximately 1.1 mg of DNA/OD <sub>600</sub> .ml)
Final quantity:	120 mg (post final ultrafiltration)
Conditioning:	1 mg/ml in 10 mM Tris, 1 mM EDTA, pH 7.5
Contaminating proteins:	< 0.20% (n=1) (*)
Contaminating RNAs:	< 1.0% (n=1) (*)
Endotoxins:	3.7 EU/mg of DNA
Functionality:	Active in transfection <i>in vitro</i>
Structure:	Conform by restriction analysis
Yield:	~30%

(\*) values < the quantification limits for the assays.



It will be understood that the term "comprises" or its grammatical variants as used in this specification and claims is equivalent to the term "includes" and is not to be taken as excluding the presence of other elements or features.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

- 1 Method for preparing large scale plasmid DNA from a wet cell biomass harvested after fermentation of a producing cell comprising said plasmid DNA, characterized in that it comprises the following steps:
- 5
- (a) alkaline lysis of the resuspended biomass, after resuspension of the wet cell biomass
  - (b) acidification at a high ionic strength,
  - (c) removal of the insoluble matter,
  - 10 (d) reduction of the endotoxins by a factor of at least 100 in the presence of a detergent,
  - (e) reduction of the ribonucleic acids (RNA),
  - (f) gel filtration chromatography after step (d), and
  - 15 (g) conditioning.
- 2 Method according to claim 1, characterized in that the alkaline lysis step is carried out in the presence of sodium hydroxide and sodium dodecyl sulfate (SDS).
- 3 Method according to claim 1 or 2, characterised in that
- 20 that the acidification step is carried out in the presence of potassium acetate at a final pH of about 5.1.
- 4 Method according to one of claims 1 to 3, characterized in that the step of removing the insoluble matter comprises at least one macrofiltration step.
- 25 5 Method according to claim 4, characterized in that it comprises two to three successive macrofiltration steps on filters of decreasing porosities below 100  $\mu\text{m}$ , 40  $\mu\text{m}$  and/or 16  $\mu\text{m}$ .
- 6 Method according to claim 4, characterized in that
- 30 it comprises a single macrofiltration step carried out on a cartridge having a porosity equal to 8  $\mu\text{m}$  or to 3  $\mu\text{m}$ .
- 7 Method according to one of claims 1 to 6, characterized in that the endotoxin reducing step comprises at least one extraction step in the presence



of a detergent having a cloud point of between 15°C and 35°C, advantageously 18°C and 30°C and preferably 20°C and 25°C.

8. Method according to Claim 7, characterized in  
5 that the endotoxin reducing step comprises 1 to 3 successive extractions carried out in the presence of 1 to 3% final of said detergent.
9. Method according to Claim 7 or 8, characterized in that the detergent is chosen from polyoxyethylenes.
- 10 10. Method according to Claim 9, characterized in that the detergent is Triton<sup>TM</sup> X-114.
11. Method according to one of Claims 7 to 10, characterized in that the endotoxin reducing step is followed by an alcohol precipitation step.
- 15 12. Method according to one of Claims 1 to 11, characterized in that the RNA reducing step comprises a selective precipitation of the RNAs under high-ionic strength conditions.
13. Method according to Claim 12, characterized in  
20 that the selective precipitation is carried out in the presence of ammonium sulfate at a final concentration of between 2 and 2.5 M.
14. Method according to Claim 12, characterized in that the selective precipitation is carried out in the  
25 presence of calcium chloride at a final concentration of between 10 mM and 2 M, advantageously between 20 mM and 0.5 M, and preferably between 50 mM and 0.1 M.
15. Method according to one of Claims 1 to 14, characterized in that the gel filtration chromatography  
30 step is carried out on a support having an exclusion limit greater than or equal to  $20 \times 10^6$  Da.
16. Method according to Claim 15, characterized in that the support is selected from the Sephacryl S500, Sephacryl S1000 and GF2000 supports.
- 35 17. Method according to one of Claims 1 to 16, characterized in that the conditioning comprises a sterilizing filtration step.
18. Method according to one of Claims 1 to 17, characterized in that it comprises, in addition,



between steps c) and d), d) and e) and/or e) and f), a concentration step optionally followed by a step of alcohol precipitation and resuspension in aqueous phase.

5 19. Method according to Claim 18, characterized in that the concentration step is carried out by ultrafiltration on a membrane having a cut-off of between 20 and 300 kDa.

10 20. Method according to Claim 19, characterized in that the concentration step is carried out by ultrafiltration on a membrane having a cut-off of between 30 and 100 kDa.

15 21. Method according to one of Claims 1 to 20, characterized in that the plasmid DNA included in said producing cell is a recombinant plasmid DNA comprising a gene selected from the genes encoding a cytokine, a cellular or nuclear receptor, a ligand, a coagulation factor, the CFTR protein, insulin, dystrophin, a growth hormone, an enzyme, an enzyme inhibitor, a polypeptide  
20 with antitumor effect, a polypeptide capable of inhibiting a bacteria, parasitic or viral infection and especially an HIV infection, an antibody, a toxin, an immunotoxin and a marker.

22. Method according to one of Claims 1 to 21 for  
25 the preparation of a plasmid DNA greater than 10 kb in size.

23. Method according to one of Claims 1 to 22, characterized in that the wet cell biomass is harvested by fermentation of an *Escherichia coli* strain  
30 comprising a recombinant plasmid DNA.

24. Method according to Claim 23, characterized in that the *Escherichia coli* strain is selected from the strains DH5, DH10B and MC1061.

25. Method according to one of Claims 1 to 24,  
35 characterized in that it comprises the following steps:

a) resuspension of the wet cell biomass,



- b) alkaline lysis of the resuspended biomass obtained in step a) in the presence of sodium hydroxide and SDS,
- 5 c) acidification of the alkaline lysate obtained in step b) in the presence of potassium acetate to a final pH of about 5.1,
- d) removal of the insoluble matter by macrofiltration of the acidified lysate obtained in step c),
- 10 e) concentration of the filtrate obtained in step d) by ultrafiltration on a membrane having a cut-off of between 20 and 300 kDa, preferably between 30 and 100 kDa, and alcohol precipitation followed by resuspension of the precipitate in aqueous medium,
- 15 f) reduction of the endotoxins in the resuspended precipitate in step e) by extraction in the presence of Triton<sup>TM</sup> X-114 followed by alcohol precipitation and resuspension of the precipitate in aqueous medium,
- 20 g) reduction of the RNAs in the resuspended precipitate obtained in step f) by selective precipitation in the presence of ammonium sulfate or calcium chloride,
- h) gel filtration chromatography of the supernatant obtained in step g) on a Sephacryl S500 support,
- 25 i) concentration of the fractions containing said plasmid DNA which are obtained in step h) by ultrafiltration on a membrane having a cut-off of between 20 and 300 kDa, preferably between 30 and 100 kDa, and alcohol precipitation, and
- 30 j) conditioning by resuspension of the precipitate obtained in step i) in a pharmaceutically acceptable buffer followed by sterilizing filtration and division into doses.

35 26. Pharmaceutical composition comprising a plasmid DNA purified by the method according to one of Claims 1 to 25.

27. Pharmaceutical composition according to Claim 26, comprising, in addition, a lipid compound.



- 28 Pharmaceutical composition according to claim 27,  
characterized in that the lipid compound is chosen  
from 5-carboxyspermylglycin dioctadecylamide (DOGS),  
3 $\beta$  [N-(N',N'-dimethylaminoethane) carbamoyl]  
5 cholesterol (DC-Chol), (2,3-dioleoylocyl-N-[2-  
(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-  
propanaminium trifluoro-acetate) (DOSPA), spermine  
cholesterol and spermidine cholesterol.
- 29 Pharmaceutical composition according to one of  
10 claims 26 to 28, comprising, in addition, an  
adjuvant capable of enhancing the transfecting power  
of said pharmaceutical composition.
- 30 Pharmaceutical composition according to claim 29,  
characterized in that said adjuvant is a neutral  
15 lipid and especially dioleoylphosphatidylethanolamine  
(DOPE).
- 31 Use of a pharmaceutical composition according to one  
of claims 26 to 30, for the preparation of a  
medicament intended for the treatment of the human  
20 or animal body by gene therapy.
- 32 A method of treatment for the human or animal body  
by gene therapy comprising administration of an  
effective amount of a pharmaceutical composition  
according to one of claims 26 to 30.
- 25 33. Method according to one of claims 1 to 6,  
characterized in that the endotoxin reducing step  
comprises at least one extraction step in the  
presence of a detergent having a cloud point of  
between 20°C and 25°C.



- 34 Method according to claim 12, characterized in that the selective precipitation is carried out in the presence of calcium chloride at a final concentration of between 50mM and 0.1M.
- 5 35 A method according to any one of claims 1 to 24 in which step (d) is conducted before step (e).
- 36 A method for preparing a plasmid DNA from a wet cell biomass harvested after fermentation of a producing cell substantially as herein described with reference to the examples.
- 10 37 A pharmaceutical composition comprising a plasmid DNA prepared according to claim 33.

15 Transgene S.A.  
By its registered patent attorneys  
Freehills Carter Smith Beadle

26 February 2001



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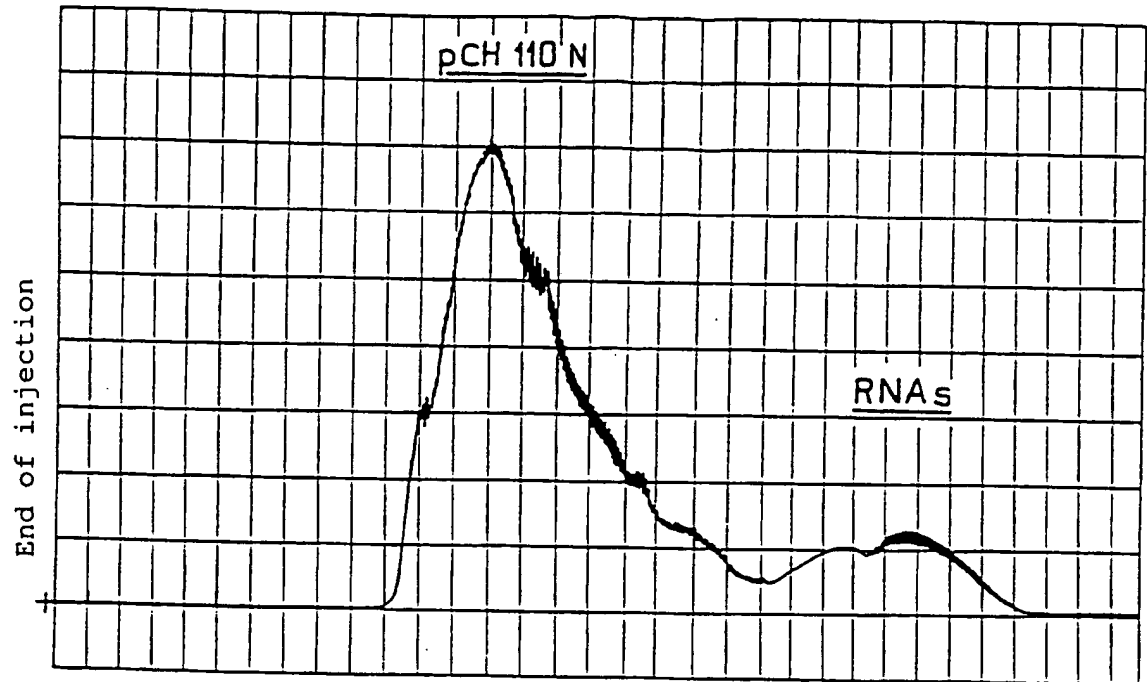
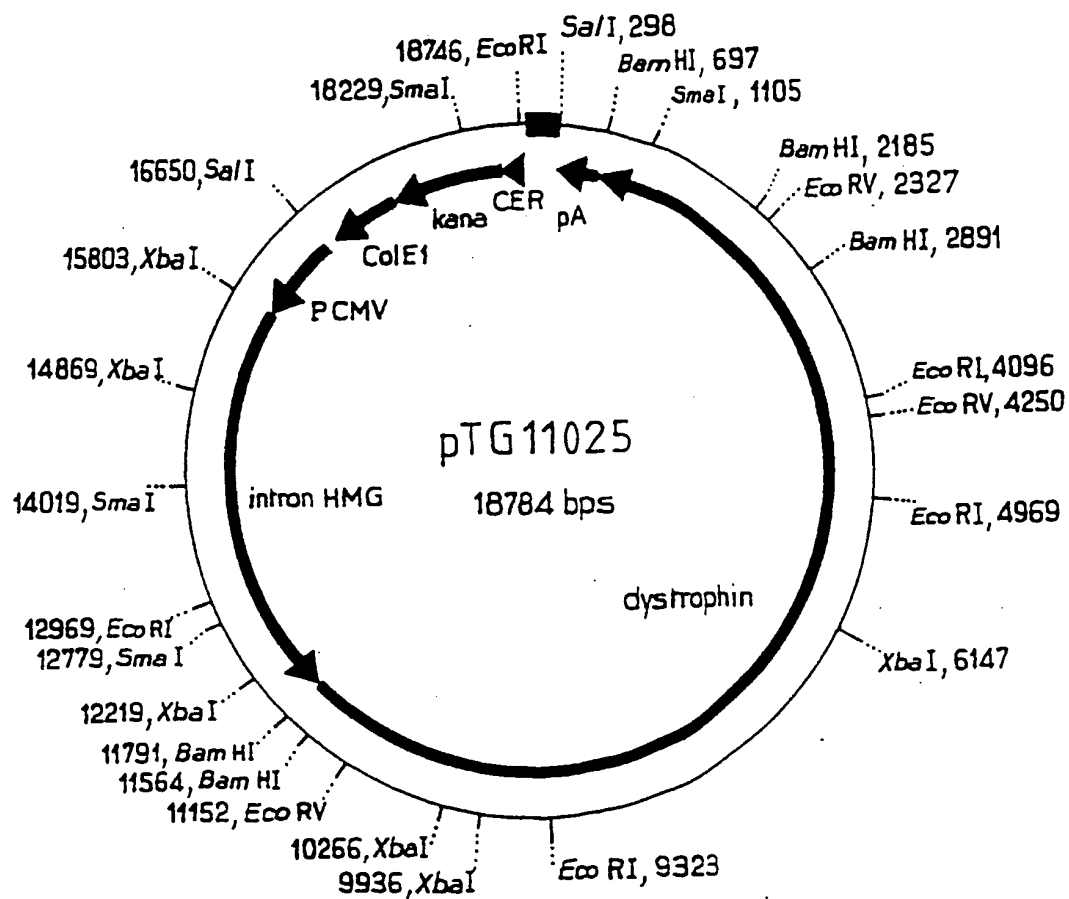


FIG. 1

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FIG. 2



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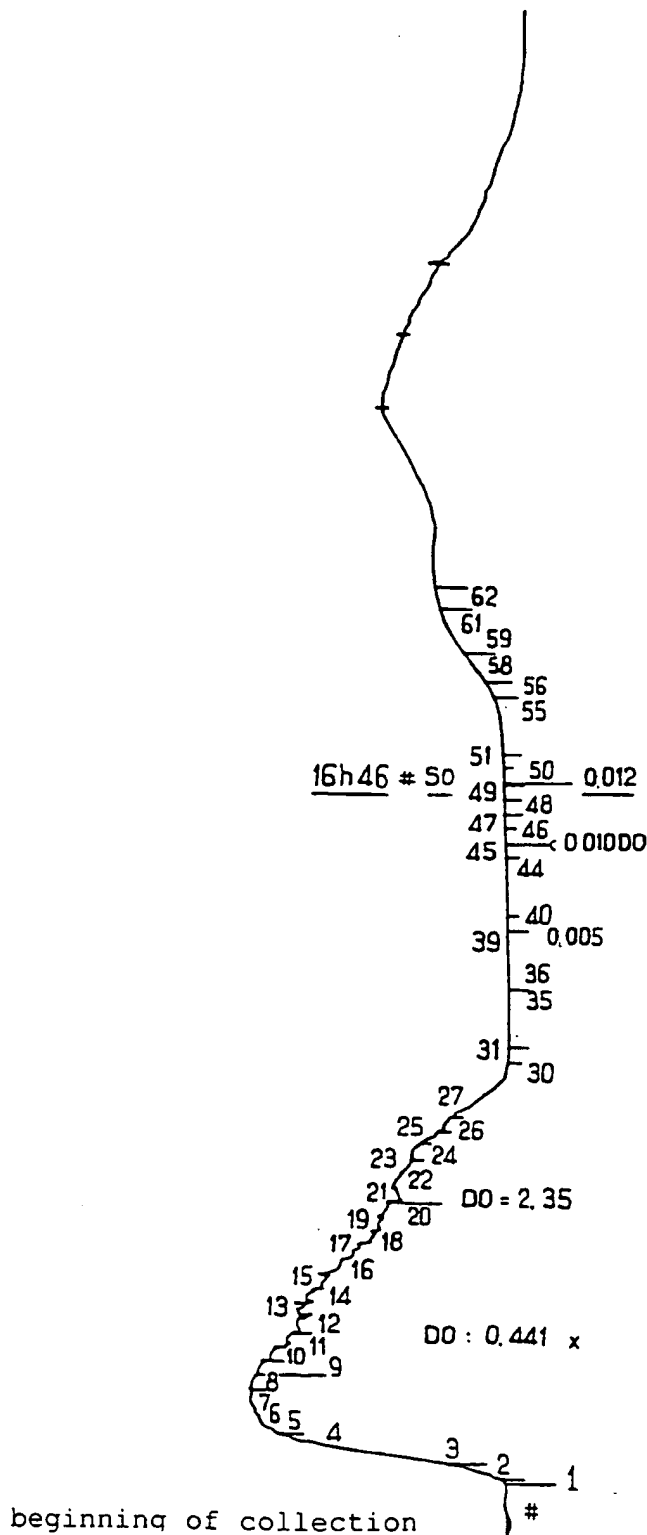
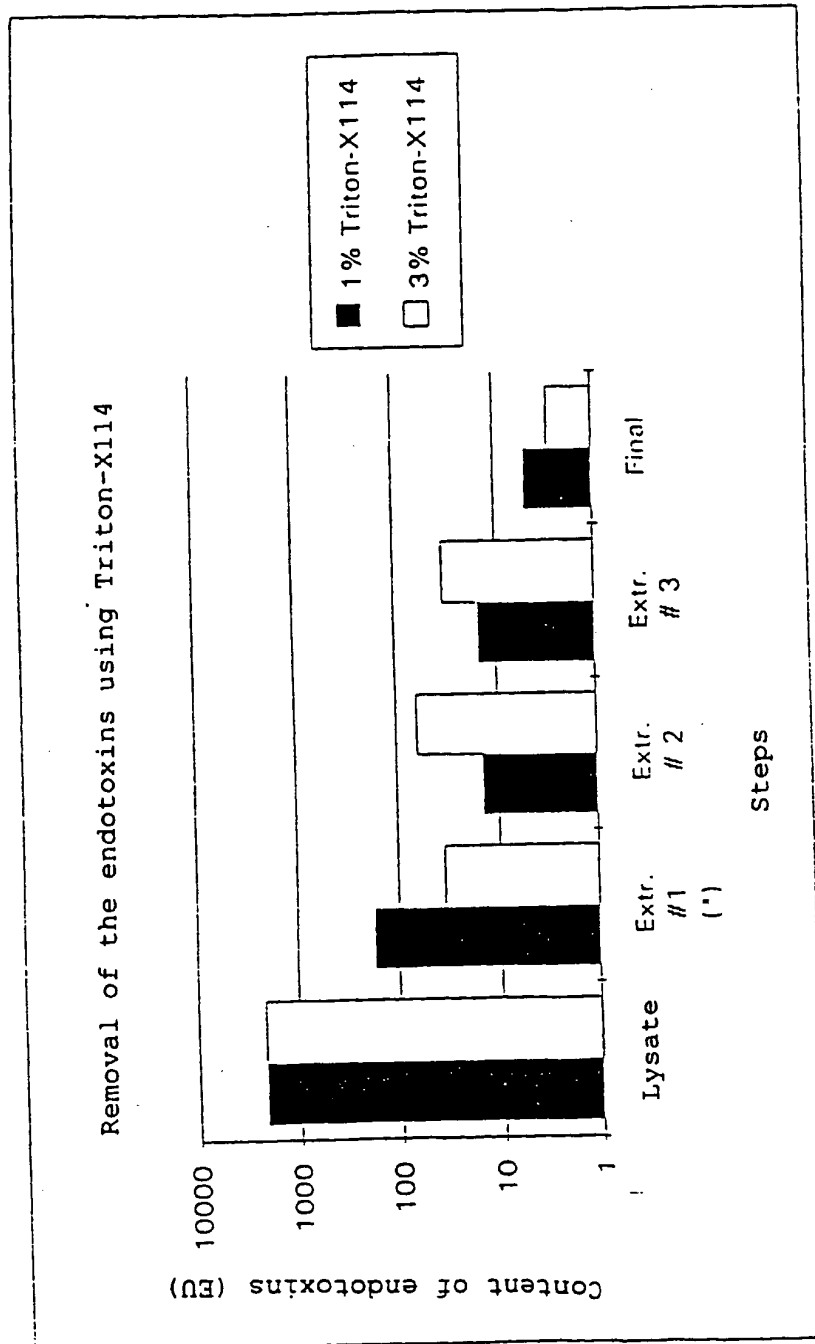


FIG.3

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Legend:

- Lysate Initial alkaline lysate
- Extr. 1 (\*) After the 1st extraction with Triton X-114
- Extr. 2 After the 2nd extraction with Triton X-114
- Extr. 3 After the 3rd extraction with Triton X-114
- Final. Final product after alcohol precipitation

FIG. 4

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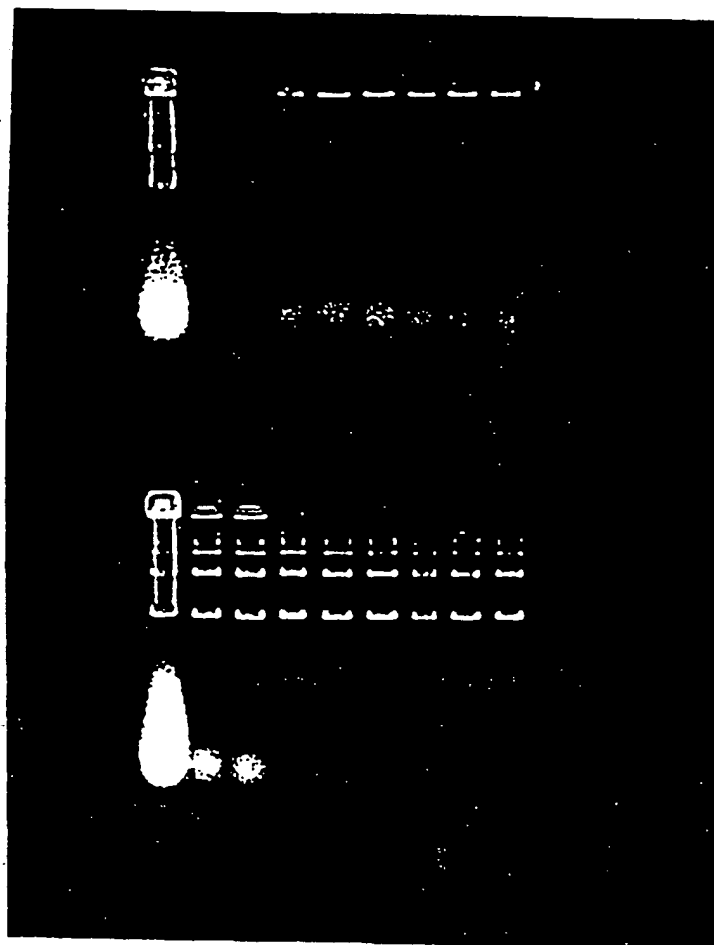
Line	Sample
1	Total alkaline lysate: overload 10 x
2	Total alkaline lysate
3	Precipitation with 0.5 M final ammonium sulfate
4	Precipitation with 1.0 M "
5	Precipitation with 1.5 M "
6	Precipitation with 2.0 M "
7	Precipitation with 2.5 M "
8	Precipitation with 3.0 M "
9	Precipitation with 3.2 M "

1 2 3 4 5 6 7 8 9

Gel No. 1:

Precipitated material  
(RNAs)

Gel No. 2:

Soluble material  
(plasmid DNA)FIG. 5

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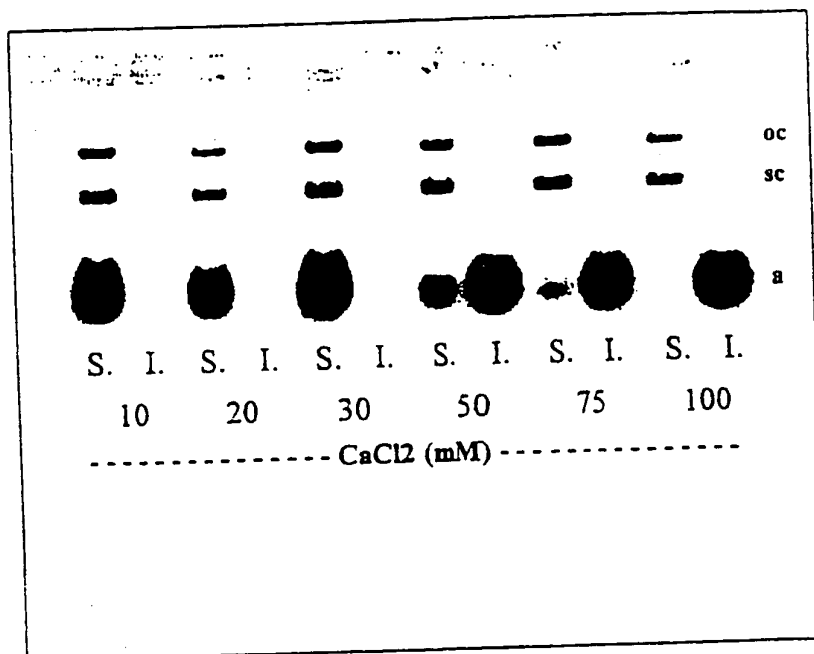


FIG. 6

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